Nur77 controls tolerance induction, terminal differentiation, and effector functions in semi-invariant natural killer T cells

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Semi-invariant natural killer T (iNKT) cells are self-reactive lymphocytes, yet how this lineage attains self-tolerance remains unknown. iNKT cells constitutively express high levels of *Nr4a1*-encoded Nur77, a transcription factor that integrates signal strength downstream of the T cell receptor (TCR) within activated thymocytes and peripheral T cells. The function of Nur77 in iNKT cells is unknown. Here we report that sustained Nur77 overexpression ($Nur77^{tg}$) in mouse thymocytes abrogates iNKT cell development. Introgression of a rearranged *Vα14-Jα18* TCR-α chain gene into the $Nur77^{tg}$ ($Nur77^{tg}$;$Vα14^{tg}$) mouse rescued iNKT cell development up to the early precursor stage, stage 0. iNKT cells in bone marrow chimeras that reconstituted thymic cellularity developed beyond stage 0 precursors and yielded IL-4–producing NKT2 cell subset but not IFN-γ–producing NKT1 cell subset. Nonetheless, the developing thymic iNKT cells that emerged in these chimeras expressed the exhaustion marker PD1 and responded poorly to a strong glycolipid agonist. Thus, Nur77 integrates signals emanating from the TCR to control thymic iNKT cell tolerance induction, terminal differentiation, and effector functions.

iNKT cells | development | function | Nur77

Semi-invariant natural killer T (iNKT) cells, which express an invariant T-cell receptor (TCR)-α chain (mouse Vα14Jα18 or human Vα24-Jα18) paired with a limited number of TCR-β chains, are innate-like T lymphocytes that respond quickly to glycolipid agonists, such as the marine sponge-derived glycosphingolipid α-galactosylceramide (αGC) (1–3). Activated iNKT cells secrete a variety of proinflammatory cytokines and chemokines by which they steer innate and adaptive immune responses to microbial antigens, autoantigens, and alloantigens to promote health or disease (1–3). iNKT cell functions are controlled by self and nonself lipid agonists presented by MHC-like CD1d molecules (1–4). This recognition of self-agonists by iNKT cells, especially in the context of sterile inflammation (5), warrants an in-depth investigation of tolerance induction mechanisms in iNKT cells.

iNKT cell precursors arise in the thymus through a developmental program that is shared with conventional T cells until the $CD4^+8^+$ double-positive (DP) stage (2, 3, 6, 7). From that point on, iNKT cell precursors undergo a unique developmental program specified by a lineage-specific gene regulatory network induced by recognition of agonistic self-lipid ligands via the semi-invariant TCR in the presence of growth factors, such as IL-7 and IL-15 (2, 3, 6–9). These signals result in progressive maturation of stage 0 precursors to stage 1 through stage 3 (2, 3, 6, 7) and further differentiation into functional NKT1, NKT2, and NKT17 subsets (3, 10–15). TCR ligation by agonistic self-lipid ligands signals iNKT cell lineage commitment, resulting in the induction of several transcription factors, including *Nr4a1*-encoded Nur77 and PLZF (promyelocytic leukemia zinc finger encoded by *Zbtb16*). While the functions of PLZF and several lineage-specific transcription factors have been studied in detail, the contribution of Nur77 to iNKT cell development and function has yet to be investigated (2, 3, 6, 7, 16).

The Nur77 family of orphan nuclear receptor transcription factors comprises three members, Nur77, Nurr1, and Nor1, which are encoded by *Nr4a1*, *Nr4a2*, and *Nr4a3* loci, respectively (17, 18). These transcription factors are related by a high degree of homology in their DNA-binding domains (17, 18). Thus, only mice lacking all three members in their T cells succumb to autoimmunity by 14 to 21 d after birth due to impaired thymic regulatory T cell (Treg) development and overt T cell autoreactivity (19). Mechanistically, Nur77 binds to the *Foxp3* gene promotor to induce *Foxp3* expression and thus the generation of Tregs (19). On the other hand, high Nur77 levels induce T cell apoptosis by converting the prosurvival factor Bcl-2 to a proapoptotic agent (20). Overexpression of *Nr4a1* ($Nur77^{tg}$) in mice under the control of the proximal *Lck* promoter abrogates conventional $CD4^+$ and $CD8^+$ T cell development, while overexpression of a dominant negative *Nr4a1* mutant, which lacks the DNA-binding domain, blocks negative selection of $CD4^+$ and $CD8^+$ cells. Taken together, these findings support a role for Nur77 in thymic negative selection (17, 20–23).

Significance

Semi-invariant natural killer T (iNKT) cells are innate-like lymphocytes that control a variety of immune functions. iNKT cell functions are mediated by high-affinity interactions with self-agonists displayed by the lipid-presenting CD1d molecule. How iNKT cells attain tolerance to high-affinity interactions with self remains unknown. This understanding is of immunologic import, as an unbridled iNKT cell-mediated response can cause inflammatory diseases. We discovered that Nur77—a transcription factor expressed at high levels in iNKT cells—induced caspase-3–mediated apoptosis and markers of T cell exhaustion such as PD-1 in iNKT cell precursors, which led to hyporesponsiveness to a high-affinity lipid agonist. Thus, Nur77 plays a central role in self-tolerance induction of iNKT cells.

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Beyond thymic development, Nur77 also controls peripheral T cell function, as Nur77 induction in peripheral T cells results in T cell exhaustion (24, 25), and the combined ablation of *Nr4a1*, *Nr4a2*, and *Nr4a3* expression results in the reversal of exhaustion and enhances T cell responsiveness against tumor cells and viral infections (24, 25). Thus, in conventional T cells, Nur77 induces tolerance through distinct mechanisms: induction of negative selection of autoreactive thymocytes, generation of Tregs, and induction of peripheral T cell exhaustion (17, 18, 20–26). Despite the high Nur77 expression in iNKT cells, its function has not been investigated to date.

A previous study found that iNKT cells in NFκB signaling-deficient IκBΔN^{tg} mice arrested at stage 0/stage 1 of development were enriched in cells expressing Nur77 (27). Subsequent studies showed a correlation between Nur77 level in iNKT cells and TCR signal strength (16, 28). As iNKT cells undergo agonist selection, stage 0 iNKT cell precursors constitutively express high levels of Nur77 (16). Among the terminally differentiated thymic iNKT cell subsets, NKT2 cells have relatively higher Nur77 levels compared with NKT1 and NKT17 subsets (11, 28, 29). IL-10–producing fat tissue-derived iNKT cells also express higher *Nr4a1* transcripts than thymic iNKT cells (14). These reports warrant an investigation of Nur77 function in iNKT cells.

It is generally assumed that the iNKT cell TCR repertoire is solely generated by positive selection resulting from the interactions of the precursors with CD1d-agonistic ligand complex and SLAM (signaling lymphocyte activation molecule) receptors on DP thymocytes (5, 30–37). Nonetheless, indirect evidence supports a role for negative selection in sculpting a functional iNKT cell TCR repertoire (38–42), potentially weeding out high-affinity self-reactive iNKT cells. These two selective processes do not explain why Nur77 expression persists at a higher level in iNKT cells compared with conventional T cells, however. Moreover, TCR signal strength controls positive and negative selection of conventional T cells, as well as the functional differentiation of both conventional T and iNKT cells (17, 19, 28).

As Nur77 level serves as a proxy for TCR signal strength, and because the role of Nur77 in iNKT cells remains unknown, we investigated iNKT cell development and function in Nur77^{tg} mice, which overexpress *Nr4a1* selectively in thymocytes. This model was used because iNKT cells develop normally in *Nr4a1*-deficient and *Nr4a1*, *Nr4a2*, and *Nr4a3* triple-deficient (TKO) mice (19, 43). The TKO mice survive for only ∼18 to 21 d and succumb to severe autoimmune disorders (19); thus, these mice do not allow for a detailed study of iNKT cell biology (44, 45).

Herein we report studies of iNKT cell development in mice that overexpress Nur77 within thymocytes. Our data reveal that Nur77 integrates signals emanating from the TCR to control thymic iNKT cell tolerance induction, terminal differentiation, and effector functions.

Results

iNKT Cells Fail to Develop in Mice Overexpressing Nur77 in Thymocytes. Mice lacking either Nur77 or all three Nur77 members (Nur77, Nor1, and Nurr1; TKO mice) develop iNKT cells (19, 43). Because the TKO mice survive for only ∼18 to 21 d and succumb to severe autoimmune disorders (19), using them to perform detailed analyses of iNKT cell biology is infeasible (44, 45). Therefore, we analyzed mice in which a *Nr4a1* transgene (Nur77^{tg}) is expressed in a thymocyte-specific manner under the control of the proximal *Lck* promoter (17). Consistent with previous reports (17, 23), we found that conventional T cell development and peripheral T cell frequency were severely reduced in Nur77^{tg} mice compared with C57BL/6 (B6) mice and nontransgenic littermates (*SI Appendix*, Fig. S1 *A* and *B*). As reported previously (21), we found that CD4$^+$CD25$^+$Foxp3$^+$ Tregs were enriched in spleen and lymph nodes of Nur77^{tg} mice, which is consistent with the high constitutive Nur77 expression in Tregs (16) and Treg resistance to Nur77-induced apoptosis (21). Because iNKT cells—like Tregs that undergo thymic agonist selection—have high constitutive Nur77 expression (16), we predicted that iNKT cells would be resistant to Nur77-induced apoptosis. Contrary to this prediction, however, we found that Nur77^{tg} mice had no iNKT cells in the thymus and periphery (*SI Appendix*, Fig. S1 *C* and *D*). Thus, akin to conventional T cells, high and sustained Nur77 levels in thymocytes abrogate iNKT cell development.

Introgression of a Rearranged Vα14-Jα18 α-Chain Gene into Nur77^{tg} Mice Partially Rescues iNKT Cell Development. Owing to overt apoptosis, DP thymocyte numbers are severely reduced in mice constitutively overexpressing Nur77 in thymocytes (17, 46). We and others have shown that iNKT cells fail to develop in mice in which the DP thymocyte survival window is shortened, as this prevents distal *Vα14* to distal *Jα18* rearrangement to encode the invariant TCR (TCR*i*) α-chain expressed by iNKT cells (3, 46–49). Therefore, we introgressed a rearranged *Vα14-Jα18* α-chain gene into Nur77^{tg} mice and evaluated iNKT cell development and function in the resulting Nur77^{tg};Vα14^{tg} mice. We found that iNKT cells developed in these Nur77^{tg};Vα14^{tg} mice, but total thymic and splenic iNKT cell numbers were significantly lower in these mice compared with Vα14^{tg} and B6 controls (Fig. 1 *A* and *B*). Further analysis revealed that iNKT cell development was arrested at stage 0 (CD24^{HI}CD44^{NEG}NK1.1^{NEG}; Fig. 1 *C* and *D*). Consistent with this early arrest, Nur77^{tg};Vα14^{tg} mice had fewer thymic CCR7^{HI}PD1^{LO} iNKT cells (downstream precursors that succeed stage 0 cells) (12) compared with Vα14^{tg} mice (*SI Appendix*, Fig. S2 *A* and *B*).

Further analyses showed that Nur77 expression was not variegated but was uniformly higher at all stages of iNKT cell development in Nur77^{tg};Vα14^{tg} mice compared with Vα14^{tg} mice (Fig. 1 *E* and *F*). Conversely, PLZF expression was significantly lower in thymic stage 0+1 iNKT cells in Nur77^{tg};Vα14^{tg} mice compared with Vα14^{tg} mice (Fig. 1 *G* and *H*). Thus, compared with Vα14^{tg} mice, Nur77^{tg};Vα14^{tg} mice had a lower percentage of PLZF$^+$ iNKT cells within thymic stage 0+1 (Fig. 1*I*). Based on this result, we analyzed the three iNKT cell subsets (NKT1, NKT2, and NKT17) identifiable by differential PLZF, T-bet, and RORγt expression (10). Consistent with reduced PLZF expression, Nur77^{tg};Vα14^{tg} mice lacked thymic NKT1 (PLZF^{INT}T-bet^{HI}), NKT2 (PLZF^{HI}T-bet^{NEG}RORγt^{NEG}), and NKT17 (PLZF^{INT-HI}RORγt$^+$) subsets (*SI Appendix*, Fig. S2 *A* and *B*), all of which showed higher Nur77 expression in Nur77^{tg};Vα14^{tg} mice compared with Vα14^{tg} mice (*SI Appendix*, Fig. S2 *C* and *D*). Collectively, these results suggest that Nur77 overexpression within thymocytes arrests iNKT cell development at a very early stage.

Peripheral iNKT Cell Development Is Altered in Nur77^{tg};Vα14^{tg} Mice. After development, thymic iNKT cells emigrate and complete maturation at peripheral sites. To investigate the effect of Nur77 overexpression, we tracked iNKT cells in the liver, lung, lymph nodes, and adipose tissue. Peripheral iNKT cell frequency was reduced in Nur77^{tg};Vα14^{tg} mice compared with Vα14^{tg} mice (Fig. 2*A* and *SI Appendix*, Fig. S3*A*). A proportion of peripheral iNKT cells matured to stages 2 and 3, but due to significantly low T cell numbers, the absolute number of mature iNKT cells in Nur77^{tg};Vα14^{tg} mice remained significantly lower in all organs compared with levels in Vα14^{tg} mice (Fig. 2*A* and *SI Appendix*, Fig. S3*A*).

The reduced number of stage 2 and 3 peripheral iNKT cells in Nur77^{tg};Vα14^{tg} mice led us to investigate iNKT cell subsets. Similar to thymic iNKT cells, the majority of peripheral iNKT cells in Nur77^{tg};Vα14^{tg} mice were either PLZF^{LO} or PLZF^{INT}, in contrast to Vα14^{tg} mice, in which the majority of peripheral iNKT cells were PLZF^{INT-HI}. Peripheral NKT1 and NKT2 cell numbers were consistently low in Nur77^{tg};Vα14^{tg} mice (Fig. 2*B* and *SI Appendix*, Fig. S3*B*). Interestingly, the peripheral NKT17

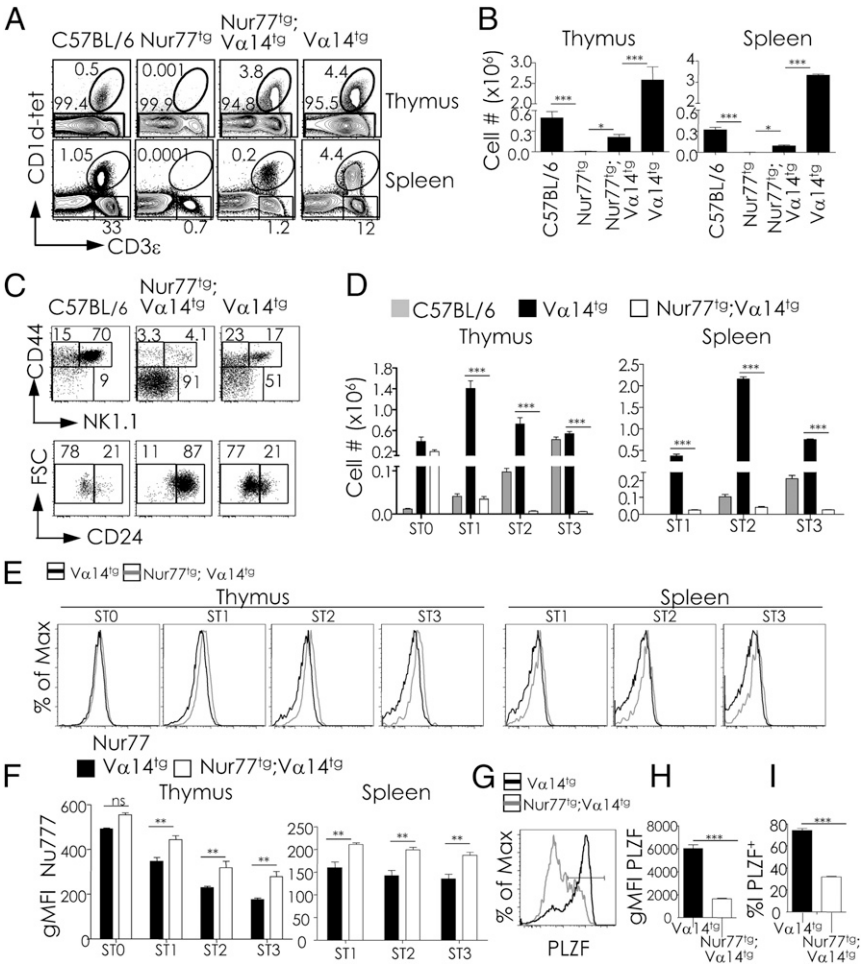

Fig. 1. Introgression of a rearranged Vα14i TCR-α chain gene partially rescues iNKT cell development in Nur77^{tg} mice. (*A*) Thymic and splenic iNKT cells from B6 (n = 6), Nur77^{tg} (n = 4), Nur77^{tg};Vα14^{tg} (n = 7), and Vα14^{tg} (n = 4) mice were identified as CD3ε^{+}tetramer^{+} (fluorophore tagged, mouse CD1d-αGC) cells within electronically gated CD8α^{LO} thymocytes or B220^{LO} splenocytes. (*B*) Absolute cell numbers were calculated from % iNKT cells and total cell counts. *n*, as in *A*. (*C*) iNKT cell developmental stages were identified as CD44^{NEG}NK1.1^{NEG} stage 0+1, CD44^{+}NK1.1^{NEG} stage 2, or CD44^{+}NK1.1^{+} stage 3 in the thymus. CD44^{NEG}NK1.1^{NEG} stage 0+1 iNKT cells were further gated to identify CD24^{HI} stage 0 or CD24$^{LO—NEG}$ stage 1 iNKT cells. Numbers are % cells among total iNKT cells. *n*, as in *A*. (*D*) Absolute numbers were calculated from the absolute iNKT cell numbers and % of stages 0, 1, 2, and 3 cells in *C*. *n*, as in *A*. Data are mean ± SEM from three independent experiments. (*E* and *F*) Expression of Nur77 on thymic stage 0 to 3 and splenic stage 1 to 3 iNKT cells from Vα14^{tg} (n = 3) and Nur77^{tg};Vα14^{tg} (n = 3) mice was determined by flow cytometry after surface staining to identify iNKT cells (as in *A*) and iNKT cell developmental stages (as in *C*) after nuclear staining with specific mAbs for Nur77. (*E*) Overlay histograms are representative of two independent experiments. (*F*) Geometric mean fluorescence intensity (gMFI) of Nur77 expression in stage 0 to 3 iNKT cells, plotted as mean gMFI ± SEM. *n* and replicates as in *E*. (*G–I*) Expression of PLZF on thymic stage 0+1 iNKT cells from Vα14^{tg} (n = 3) and Nur77^{tg};Vα14^{tg} (n = 4) mice as determined by flow cytometry after surface staining to identify iNKT cells as in *A*, and iNKT cell developmental stages after nuclear staining with specific mAb for PLZF as in *C*. (*G*) Overlay histograms are representative of two independent experiments. (*H*) gMFI of PLZF expression in thymic stage 0+1 iNKT cells plotted as mean gMFI ± SEM. *n* and replicates as in *G*. (*I*) Percentages of thymic PLZF^{HI} stage 0+1 iNKT cells is plotted as mean percentages ± SEM; *n* as in *G*. ns, not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$.

subset was enriched in the spleen, liver, and lung of Nur77^{tg};Vα14^{tg} mice to numbers similar to those seen in Vα14^{tg} mice (Fig. 2*B* and *SI Appendix*, Fig. S3*B*). Thus, akin to thymic iNKT cells, Nur77 overexpression alters peripheral iNKT cell development and subset differentiation.

CD1d and SLAM Levels in DP Thymocytes and Endogenous Antigen-Presenting Function Are Intact in Nur77^{tg};Vα14^{tg} Mice. RORγt expression is required for DP thymocyte survival and *Vα14*-to-*Jα18* gene rearrangement. Assessment of RORγt expression showed higher levels in DP thymocytes of Nur77^{tg};Vα14^{tg} mice compared with those of Vα14^{tg} mice (*SI Appendix*, Fig. S4 *A* and *B*). Unlike conventional T cells that are positively selected by the MHC on thymic epithelial cells, iNKT cell positive selection depends on interactions of the precursors with CD1d and SLAM receptors on DP thymocytes (1, 3–6, 30–37). We found that Nur77^{tg};Vα14^{tg} mouse and Vα14^{tg} mouse thymocytes expressed similar levels of CD1d. Expression levels of CD48 (SLAMF2), Ly9 (SLAMF3), CD84 (SLAMF5), and CD319 (SLAMF7) on DP thymocytes and of SLAM (SLAMF1), CD48 (SLAMF2), and Ly108 (SLAMF6) on stage 0 iNKT cells were significantly higher in Nur77^{tg};Vα14^{tg} mice compared with Vα14^{tg} mice (*SI Appendix*, Fig. S4 *C–H*). Thus, we predict that CD1d^{+} self-agonist–TCR and SLAM–SLAM interactions are intact in Nur77^{tg};Vα14^{tg} mice and are consistent with developmental arrest after positive selection.

To ascertain whether CD1d expressed on DP thymocytes was functional and presented the endogenous lipid agonists, FACS-sorted DP thymocytes from Nur77^{tg};Vα14^{tg} and Vα14^{tg} mice were cocultivated in vitro with Vα14^{+} NKT cell hybridomas as described previously (50, 51). This coculture yielded similar levels of IL-2 from Vα14^{+} NKT cell hybridomas only when stimulated by DP thymocytes from both Nur77^{tg};Vα14^{tg} and Vα14^{tg}

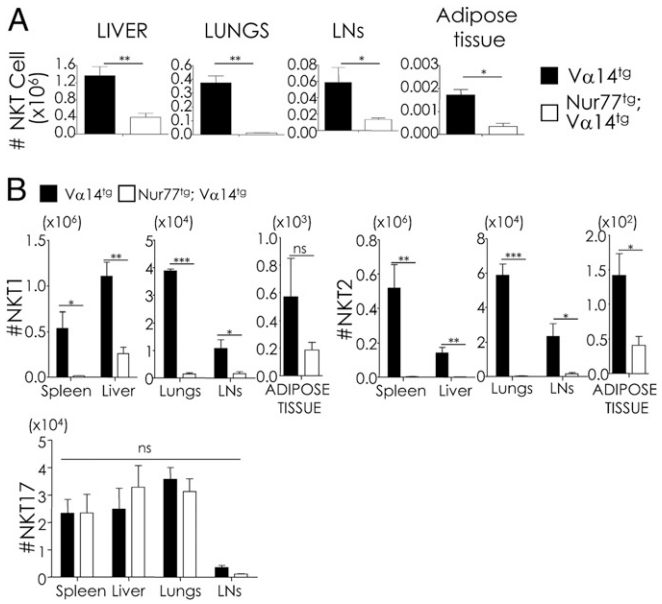

Fig. 2. Nur77 overexpression alters peripheral iNKT cells in Nur77^{tg};Vα14^{tg} mice. (*A*) iNKT cells in indicated organs from Nur77^{tg};Vα14^{tg} ($n = 3$) and Vα14^{tg} ($n = 3$) mice were identified as in Fig. 1*A*. Absolute iNKT cell numbers were calculated using counting beads (*Materials and Methods*). Data are mean ± SEM from two independent experiments. (*B*) Following surface staining, iNKT cells were stained with specific mAbs for PLZF, T-bet, and RORγt. iNKT cell subsets were identified as PLZF^{int}Tbet^{hi} NKT1, PLZF^{hi} NKT2, and PLZF^{hi}RORγt^{+} NKT17 cells. Absolute numbers were calculated from absolute NKT cell numbers and NKT1, NKT2, or NKT17 cell frequencies (representative flow plots shown in *SI Appendix*, Fig. S3*B*). Data are mean ± SEM from two independent experiments. *n*, as in *A*. ns, not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$.

mice (*SI Appendix*, Fig. S4*I*). Taken together, these findings indicate that DP thymocytes express functional CD1d and can engage in SLAM–SLAM interactions even when these cells overexpress Nur77.

iNKT Cells in Nur77^{tg};Vα14^{tg} Mice Recognize αGC but Are Functionally Impaired. Intact TCR signaling is critical for iNKT cell intrathymic expansion and developmental progression (28, 52–55). Nur77 is an immediate early gene induced downstream of TCR signaling (16, 17, 19, 22). To assess whether iNKT cell TCR signaling was impaired, the Nur77^{GFP} reporter transgene (16) was introgressed into Nur77^{tg};Vα14^{tg} (Nur77^{tg};Vα14^{tg};Nur77^{GFP}) and Vα14^{tg} (Vα14^{tg};Nur77^{GFP}) mice, and constitutive and inducible Nur77^{GFP} expression were investigated. Higher constitutive Nur77^{GFP} expression was seen in thymic iNKT cells of Nur77^{tg};Vα14^{tg};Nur77^{GFP} mice compared with those of Vα14^{tg};Nur77^{GFP} mice, with similar Nur77^{GFP} expression in stage 0 iNKT cells but higher expression in stage 1 to 3 iNKT cells (Fig. 3 *A* and *B*). Although there was a trend toward higher constitutive Nur77^{GFP} expression in splenic iNKT cells from Nur77^{tg};Vα14^{tg};Nur77^{GFP} mice, this was not statistically significant (Fig. 3 *C* and *D*). Importantly, there was no difference in inducible Nur77^{GFP} expression on αGC stimulation in vivo (Fig. 3 *C* and *D*). Thus, a high Nur77 level does not alter agonistic glycolipid-induced proximal TCR signaling in iNKT cells.

iNKT cells in Nur77^{tg};Vα14^{tg} mice poorly up-regulated CD69 in response to in vivo αGC stimulation compared with Vα14^{tg} mice (Fig. 3 *E* and *F*). Consistent with the low CD69 up-regulation, levels of secreted cytokines, such as IFN-γ and IL-4, were also low in response to in vivo stimulation of Nur77^{tg};Vα14^{tg} mouse iNKT cells with αGC (Fig. 3*G* and *SI Appendix*, Fig. S5*A*). The lower iNKT cell numbers in Nur77^{tg};Vα14^{tg} mice could have resulted in a weaker serum cytokine response. However, compared with iNKT cells in Vα14^{tg} mice, those in Nur77^{tg};Vα14^{tg} mice had lower frequencies of IFN-γ^{+}, IL-4^{+}, and IFN-γ^{+}IL-4^{+} cells in response to in vivo αGC stimulation (*SI Appendix*, Fig. S5 *B* and *C*). Considering these findings, we conclude that although TCR proximal signaling events remained intact, functional responsiveness to αGC stimulation was impaired when iNKT cells expressed high Nur77 levels.

Nur77 level controls PD1 expression in CD8^{+} tumor-infiltrating lymphocytes (26). Consistent with that finding, PD1 expression by iNKT cells was uniformly higher in Nur77^{tg};Vα14^{tg} mice compared with those in Vα14^{tg} mice. PD1 expression was uniformly high within all stages and subsets of Nur77^{tg};Vα14^{tg} mouse iNKT cells (Fig. 3 *H* and *I* and *SI Appendix*, Fig. S6 *A–D*). Collectively, the foregoing results suggest that Nur77 plays a critical role in iNKT cell development, differentiation, and function.

Nur77 Has Cell Intrinsic and Extrinsic Effects on iNKT Cell Development. To restore thymic cellularity and ascertain whether Nur77 has cell-intrinsic, cell-extrinsic, or both roles in iNKT cell development and function, we generated radiation bone marrow (BM) chimeras in iNKT cell-deficient Jα18$^{-/-}$ mice. Donor BM from Nur77^{tg};Vα14^{tg} mice, either alone or mixed at a 1:1 ratio with BM from Jα18$^{-/-}$ mice, was transferred into irradiated Jα18$^{-/-}$ mice. BM chimeras from donor B6, Jα18$^{-/-}$, Nur77^{tg}, or Vα14^{tg}, transferred alone or mixed with Jα18$^{-/-}$ BM, served as controls. As expected, no thymic iNKT cells developed in single and mixed BM chimeras composed of Nur77^{tg} BM cells and were not detected in the periphery (Fig. 4*A* and *SI Appendix*, Fig. S7*A*). Chimeras composed of Nur77^{tg};Vα14^{tg} BM cells alone developed iNKT cells that were arrested at stage 0 (*SI Appendix*, Fig. S7*A*). Surprisingly, however, Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras developed thymic iNKT cells that progressed beyond stage 0 and populated the periphery but showed incomplete progression to stage 3 (Fig. 4 *A–C* and *SI Appendix*, Fig. S7*A*). Nonetheless, iNKT cell numbers remained consistently and significantly lower than those in control Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras (Fig. 4*A*). As well, splenic NK1.1^{NEG} and NK1.1^{+} iNKT cell numbers were reduced in Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras compared with Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras (Fig. 4*B* and *SI Appendix*, Fig. S7*A*). Consistent with iNKT cell developmental progression beyond stage 0, PLZF expression was restored to normal levels (*SI Appendix*, Fig. S7*B*).

Thus restoration of PLZF expression prompted a close study of thymic and splenic iNKT cell subsets. Owing to low total thymic and splenic iNKT cell numbers, the absolute numbers of the three iNKT cell subsets were reduced in Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras compared with Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras. Notably, of the three subsets, NKT1 cells were most severely reduced, and consistent with this arrest, iNKT cells expressed low CD122 levels (Fig. 4*C* and *SI Appendix*, Fig. S7 *B* and *C*). Progression of iNKT cells beyond stage 0 in mixed chimeras suggests an iNKT cell extrinsic role of Nur77 in blocking iNKT cell development. Nonetheless, the incomplete terminal maturation and differentiation to NKT1 cells suggest an additional, cell-intrinsic role for Nur77 in iNKT cell development and differentiation.

The use of congenically marked B6 donor BM instead of Jα18$^{-/-}$ BM generated identical results. In addition, irrespective of the numbers of Jα18$^{-/-}$ BM cells used to generate mixed chimeras, iNKT cell maturation was consistently impaired (*SI Appendix*, Fig. S8 *A–D*). This result discounts faulty encounters of iNKT cell precursors with positively selecting DP thymocytes as an explanation for impaired iNKT cell development. Taken together, the results from BM chimeras indicate that Nur77 plays cell-intrinsic and -extrinsic roles in iNKT cell development and differentiation.

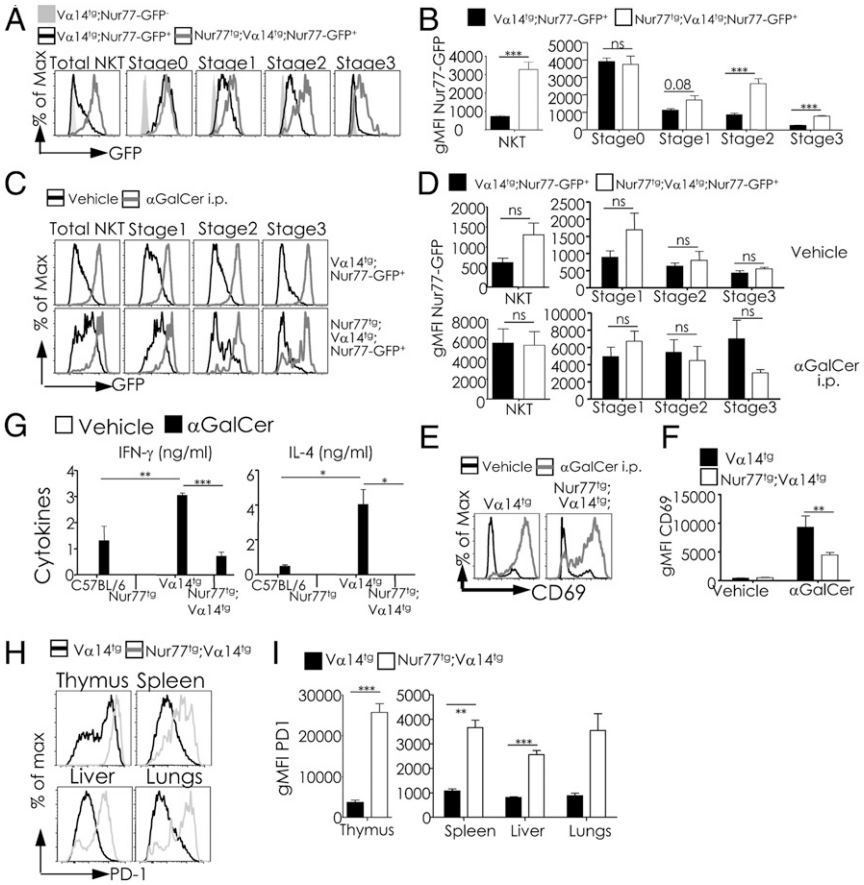

Fig. 3. Nur77^{tg};Vα14^{tg} mouse iNKT cells recognize αGC but do not respond. (*A* and *B*) Thymic iNKT cells and iNKT cell developmental stages in Vα14^{tg};Nur77^{GFP} ($n = 6$) and Nur77^{tg};Vα14^{tg};Nur77^{GFP} ($n = 6$) mice were identified as in Fig. 1, and constitutive Nur77 expression was monitored by evaluating GFP levels. Vα14^{tg};Nur77$^{GFP(NEG)}$ mice served as a negative control for background GFP expression. (*A*) Overlay histograms are representative of three independent experiments. (*B*) gMFI of Nur77^{GFP} expression by iNKT cells and its developmental stages. Data are mean ± SEM. *n* as in *A*. (*C* and *D*) Constitutive (vehicle) and induced (αGC) Nur77^{GFP} expression in splenic iNKT cells and iNKT cell developmental stages after i.p. injection with vehicle or αGC. iNKT cells were identified as in Fig. 1. (*C*) Overlay histograms are representative of three independent experiments. Vα14^{tg};Nur77^{GFP}: vehicle, $n = 3$; αGC, $n = 3$; Nur77^{tg};Vα14^{tg};Nur77^{GFP}: vehicle, $n = 3$; αGC, $n = 3$. (*D*) gMFI of Nur77^{GFP} expression in iNKT cells and iNKT cell developmental stages. Data are mean ± SEM. *n* as in *C*. (*E* and *F*) Splenic iNKT cells, identified as in Fig. 1, from Vα14^{tg} and Nur77^{tg};Vα14^{tg} mice injected i.p. with vehicle or αGC were evaluated for cell surface CD69 expression at 6 h postinjection. Overlay histograms are representative of three or four independent experiments. Vα14^{tg};Nur77^{GFP}: vehicle, $n = 4$; αGC, $n = 4$; Nur77^{tg};Vα14^{tg};Nur77^{GFP}: vehicle, $n = 4$; αGC, $n = 4$. (*F*) gMFI of CD69 expression by iNKT cells and iNKT cell developmental stages. Data are mean ± SEM. *n* as in *E*. (*G*) αGC or vehicle control was injected i.p. into B6, Nur77^{tg}, Nur77^{tg};Vα14^{tg}, and Vα14^{tg} mice as above. At 6 h postinjection, serum was harvested, and cytokines were measured using Meso Scale Discovery detection kits. Data are cumulative mean ± SEM from two independent experiments. B6: vehicle, $n = 2$; αGC, $n = 2$; Nur77^{tg}: vehicle, $n = 2$; αGC, $n = 2$; Nur77^{tg};Vα14^{tg}: vehicle, $n = 2$; αGC, $n = 4$; Vα14^{tg}: vehicle, $n = 2$; αGC, $n = 3$. (*H* and *I*) Vα14^{tg} ($n = 3$) and Nur77^{tg};Vα14^{tg} ($n = 3$) mouse iNKT cells within the indicated organs were identified as in Fig. 2, and constitutive PD-1 expression was monitored. (*H*) Overlay histograms are representative of three or four independent experiments. (*I*) gMFI of PD-1 expression by iNKT cells. Data are mean ± SEM. *n* as in *H*. ns, not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$.

Sustained Nur77 Expression Impairs iNKT Cell Survival but Not Proliferation. High Nur77 expression has been shown to induce apoptosis in conventional T cells (17). Consistent with that finding, we found enhanced caspase 3 activation in stage 1 to 3 thymic and stage 1 and 2 splenic iNKT cells from Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed BM chimeras. Whilst differences in caspase 3 activation was statistically significant in stage 2 and 3 thymic and stage 1 and 2 splenic iNKT cells, the trend toward high activated caspase 3 was also observed in stage 1 thymic iNKT cells (Fig. 5*A* and *SI Appendix*, Fig. S9*A*). Because Nur77 also inhibits T cell proliferation (56, 57), EdU (5-ethynyl-2-deoxyuridine) incorporation by iNKT cells was measured in the various chimeric mice, but no difference was observed (Fig. 5*B* and *SI Appendix*, Fig. S9*B*). Thus, Nur77 alters iNKT cell homeostasis by enhancing apoptosis throughout all stages of development without impacting proliferative capacity.

iNKT Cells from Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ Mixed Chimeras Are Exhausted. Recent studies have identified Nur77 as a mediator of T cell exhaustion (24–26). Because iNKT cells in Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras develop beyond stage 0, we tested whether these cells gain functional capacity. Serum and intracellular IFN-γ and IL-4 production by iNKT cells in response to in vivo αGC stimulation was low in Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras compared with Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras at all time points tested (Fig. 6 *A* and *B* and *SI Appendix*, Fig. S10 *A* and *B*). Thus, despite partial developmental rescue, the sustained Nur77 level in iNKT cells impairs its function in a cell-intrinsic manner.

The functional unresponsiveness described above is reminiscent of iNKT cell anergy (13, 51, 58–66) and T cell exhaustion (19, 24–26, 67). On further examination, we found, similar to Nur77^{tg};Vα14^{tg} mice, PD1 expression was significantly upregulated on iNKT cells from Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras and was consistently higher than that in iNKT cells

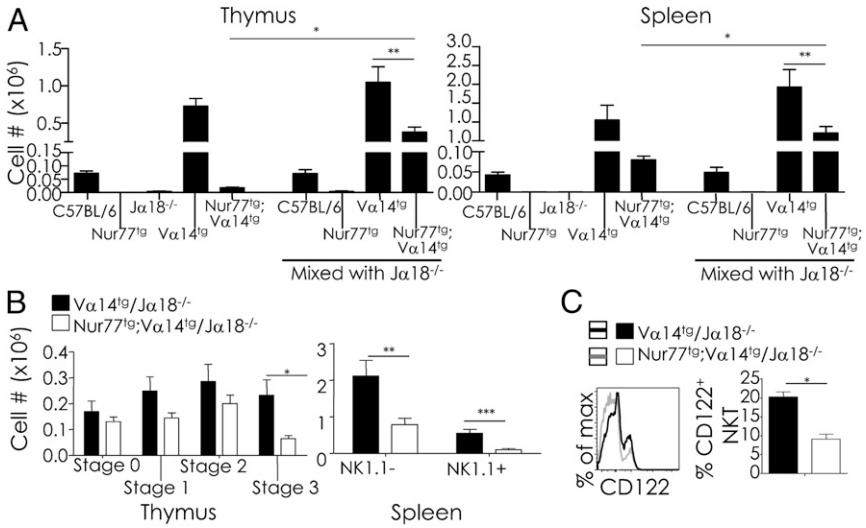

Fig. 4. Impaired iNKT cell development in Nur77^{tg};Vα14^{tg} mice is due to cell-intrinsic and cell-extrinsic defects. (*A–C*) Analysis of chimeras generated with BM cells from donor B6, Nur77^{tg}, Jα18$^{-/-}$, Nur77^{tg};Vα14^{tg}, or Vα14^{tg} mice transferred into irradiated Jα18$^{-/-}$ recipients alone (single BM chimeras) or mixed with BM from Jα18$^{-/-}$ donor at 1:1 ratio (mixed BM chimeras). (*A*) Thymic and splenic iNKT cells from B6 ($n = 6$), Nur77^{tg} ($n = 6$), Jα18$^{-/-}$ ($n = 4$), Nur77^{tg};Vα14^{tg} ($n = 6$), and Vα14^{tg} ($n = 6$) single BM chimeras and B6 ($n = 5$), Nur77^{tg} ($n = 5$), Nur77^{tg};Vα14^{tg} ($n = 25$), and Vα14^{tg} ($n = 20$) mixed BM chimeras were identified as in Fig. 1. iNKT cell developmental stages were identified as stage 0+1, stage 2, or stage 3 in thymus and spleen. In the thymus, stage 0+1 cells were further gated to distinguish stage 0 from stage 1 iNKT cells. (*A*) Absolute cell numbers were calculated from % iNKT cells and total cell counts. Data are mean ± SEM from three to five independent experiments. *n* as in *A*. (*B*) Absolute numbers of thymic stages 0, 1, 2, and 3 iNKT cells, calculated from the absolute iNKT cell numbers and % stage 0, 1, 2, and 3 cells, respectively. Representative flow plots are shown in *SI Appendix*, Fig. S7*A*. Absolute numbers of splenic NK1.1^{NEG}tetramer^{+} and NK1.1^{+}tetramer^{+} iNKT cells were calculated from the absolute iNKT cell numbers of NK1.1^{NEG}tetramer^{+} and NK1.1^{+}tetramer^{+} cells, respectively. Representative flow plots are shown in *SI Appendix*, Fig. S7*A*. Data are mean ± SEM from three to five independent experiments. *n*, as in *A*. (*C*) CD122 expression on thymic iNKT cells in Vα14^{tg} ($n = 3$) and Nur77^{tg};Vα14^{tg} ($n = 3$) mixed BM chimeras were identified as above. (*Left*) Overlay histograms are representative of two independent experiments. (*Right*) Bar graphs showing % CD122^{+} iNKT cells. Data are mean ± SEM from two independent experiments. ns, not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$.

from Vα14^{tg}/Jα18$^{-/-}$ mixed BM chimeras. PD1 expression was high in PLZF^{HI} NKT2, PLZF^{INT} NKT1, and PLZF^{LO} iNKT cell subsets (Fig. 6 *C–E*). The PLZF^{HI} population consists of CCR7^{HI}PD1^{LO} cells, a precursor to all iNKT cell subsets, and CCR7^{LO}PD1^{HI} NKT2 cells (12). PD1 was induced as early as the CCR7^{HI}PD1^{LO} thymic and splenic iNKT cells (Fig. 6 *C–E*). Furthermore, markers of IL-10–producing Tregs and NKT10 cells, FR4 and NRP1, as well as Ly108 and CD244.2 (13), were also up-regulated in thymic and splenic iNKT cells from Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras compared with these cells in Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras (*SI Appendix*, Fig. S11 *A–C*). Thus, sustained Nur77 levels induce an exhausted phenotype in iNKT cells.

Sustained Nur77 Expression Induces Foxp3 Expression in a Subset of iNKT Cells. Consistent with the report that Nur77 family members drive *Foxp3* gene expression and Treg development (19), we found increased basal Foxp3 and CD25 levels in iNKT cells from Nur77^{tg};Vα14^{tg} mice compared with those from Vα14^{tg} mice (Fig. 7 *A–D*). Thus, thymic and splenic CD25^{NEG}Foxp3^{+} and CD25^{+}Foxp3^{NEG}, but not CD25^{+}Foxp3^{+}, NKT cells emerged in Nur77^{tg};Vα14^{tg} mice that were absent in B6 mice but present in minute proportions in Vα14^{tg} mice (Fig. 7 *A*, *B*, and *E* and *SI Appendix*, Fig. S12*A*). This result is in agreement with higher proportions of thymic CD4^{+}CD25^{+}Foxp3^{NEG} and splenic CD4^{+}CD25^{NEG}Foxp3^{+} conventional T cells in Nur77^{tg};Vα14^{tg} mice (Fig. 7*E* and *SI Appendix*, Fig. S12*A*). Importantly, splenic Foxp3^{NEG} and Foxp3^{+} iNKT cells up-regulated Nur77^{GFP} and CD25 in response to in vivo αGC stimulation (Fig. 7 *F–H* and *SI Appendix*, Fig. S12*A*). It is possible that the Foxp3^{+} iNKT cells arose in Nur77^{tg};Vα14^{tg} mice because these mice lack NKT1 and NKT2 subsets; however, this is unlikely, because the frequencies of Foxp3^{+} iNKT cells were similar in Nur77^{tg};Vα14^{tg}/B6 and Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed chimeras (*SI Appendix*, Fig. S12 *B–D*). Thus, sustained Nur77 drives Foxp3 expression in a subpopulation of iNKT cells in a cell-intrinsic manner.

Discussion

This study investigated how iNKT cells attain tolerance to self-lipid agonists. Understanding the mechanisms of self-tolerance in this lineage is of immunologic import, because iNKT cells are self-reactive and mediate microbial and sterile inflammation. An unbridled inflammatory response could then lead to a chronic state that initiates and perpetuates inflammatory diseases. Thus, it is imperative that the immune system ensures that iNKT cells do not go rogue and cause autoimmune or overt auto-inflammatory responses. This study focused on Nur77 as a potential candidate transcription factor that controls tolerance induction in iNKT cells because (i) Nur77 is a proxy for TCR signal strength, (ii) Nur77 is expressed at high levels within developing iNKT cells and wanes through differentiation, (iii) the role of Nur77 in iNKT cells has not yet been defined, and (iv) Nur77 has been implicated in the negative selection of thymocytes and tolerance induction in peripheral, but not thymic, conventional T cells. In contrast to this view, Nur77 was found to be central to self-tolerance induction in developing thymic iNKT cells, making these cells hyporesponsive to agonistic stimulation in the periphery. Nur77 also was found to control effector differentiation and functions of iNKT cells, features that distinguish them from conventional T cells emerging from the thymus.

iNKT cell development was intact in mice lacking Nur77 alone or all three Nur77 family members, but a detailed analysis of iNKT cell differentiation was not possible because the TKO mice succumbed to disseminated autoimmune diseases (19, 43). Thus, we investigated iNKT cell development, differentiation, and function in mice expressing a Nur77 transgene within thymocytes

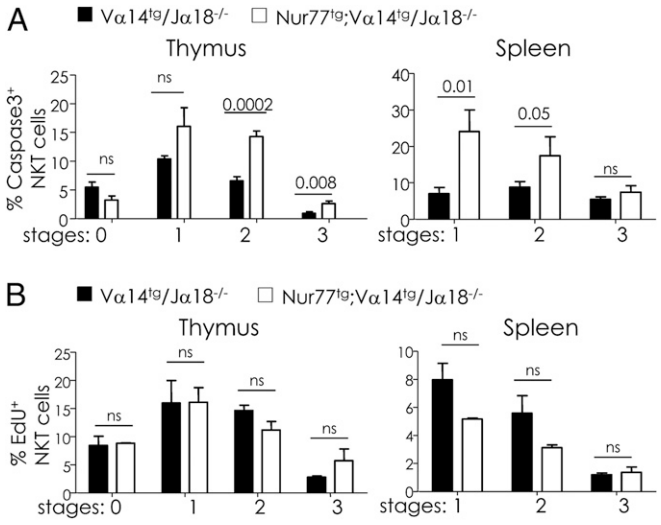

Fig. 5. Enhanced iNKT cell apoptosis in Nur77^{tg};Vα14^{tg} mixed BM chimeras. (*A*) Thymocytes and splenocytes from Vα14^{tg}/Jα18$^{-/-}$ (n = 5) and Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ (n = 5) mixed BM chimeras were treated with fluorescent-tagged active caspase-3 or control substrates. After 4 h, iNKT cells were identified, staged and caspase-3 incorporation monitored. Bar graphs depict mean ± SEM of the proportion of caspase-3$^+$ iNKT cells at different stages of maturation in the thymus (*Left*) and spleen (*Right*). (*B*) Vα14^{tg}/Jα18$^{-/-}$ (n = 3) and Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ (n = 3) mixed BM chimeras were injected i.p. with 1 mg EdU or PBS (control) and stained for EdU incorporation in iNKT cells at various developmental stages. Bar graphs depict mean ± SEM of the proportion of EdU$^+$ iNKT cells at different stages of maturation in the thymus (*Left*) and spleen (*Right*). ns, not significant (P > 0.05); *$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$.

under control of the proximal *Lck* promotor. We found that Nur77 mediated caspase-3–dependent cell death in this T cell lineage but did not alter the proliferative capacity of developing iNKT cells. The noted cell death is reminiscent of Nur77-mediated negative selection of conventional T cells (17–20). This result suggests that Nur77 mediates the pruning of pathogenic, self-reactive cells from the functional repertoire. Consistent with this view, iNKT cells were depleted by the addition of exogenous αGC to 16.5-d fetal thymic organ cultures established from wild-type mice or by repeated αGC injections into neonatal mice (40, 42). Thus, both positive and negative selection likely sculpt the functional iNKT cell repertoire.

Results from mixed BM chimera experiments suggest both cell-extrinsic and -intrinsic roles for Nur77 in iNKT cell development. Cell-extrinsic factors that affect iNKT cell development could involve defects in CD1d-restricted selecting ligand presentation, SLAM coreceptor help, and DP thymocyte and thymic medullary cellularity/architecture. The integrity of the thymic medullary compartment relies on RANK-L (receptor activator of nuclear factor-κB ligand or TNFSF11) produced by NKT2 and NKT17 cells (12). As the B6 partner in the mixed Nur77^{tg};Vα14^{tg}/B6 chimeras developed functional iNKT cell subsets, RANK-L so produced also could have "helped" iNKT cell development from the Nur77^{tg};Vα14^{tg} partner, but it did not. Thus, the thymic medullary environment makes little if any contribution to Nur77-mediated impaired iNKT cell development and differentiation. The foregoing findings also suggest that the cell-extrinsic role of Nur77 is DP thymocyte-mediated and independent of the nature of the endogenous agonist and its presentation by DP thymocytes originating from Nur77-expressing mice.

Homotypic SLAM–SLAM interactions between iNKT cell precursors and DP thymocytes are critical for the selection and differentiation of this lineage (5, 30–37, 68). Thus, this interaction is fine-tuned at the level of SLAM family member expression on the two cell types (69). We discovered that Nur77 overexpression induced PD-1 expression in stage 0 iNKT cell precursors. The sustained Nur77 expression and PD-1$^+$ phenotype of iNKT cell subsets render these cells unresponsive to the strong agonist, αGC. This phenotype was recapitulated by iNKT cells that lacked all six SLAM family receptors (70). These findings together lend to a model in which strong and persistent TCR signaling after positive selection of NKT cells induces high Nur77 expression. Because Nur77 induces apoptosis, stage 0 precursors can become dead end cells, as seen in iNKT cells that overexpress Nur77. Instead, the induction of SLAM family receptor signaling overrides Nur77-induced apoptosis and facilitates continued iNKT cell development. Consistent with this model, increased SLAM (SLAMF1) and Ly108 (SLAMF6) expression in stage 0 iNKT cells could not temper the effects of Nur77, perhaps because Nur77 overexpression was driven by the *Lck* proximal promotor rather than the native *Nr4a1* promotor. The next step would be to elucidate how the SLAM-SAP-Fyn signaling module controls Nur77 expression and function.

Even though the majority of NKT cells were arrested at the thymic precursor stage 0 in Nur77^{tg};Vα14^{tg} mice, a few thymic NKT1, NKT2, and NKT17 cells with a mature phenotype escaped this maturation arrest. Furthermore, although the periphery lacked NKT1 and NKT2 subsets, NKT17 development was unaffected. Based on CD5, CD6, and Ly6C expression patterns, a recent study reported that differentiation of the three iNKT subsets required distinct TCR signal strengths: NKT2 > NKT17 > NKT1 (28). Consistent with this hierarchy, Nur77 expression correlated with TCR signal strength integrating NKT cell activation to downstream differentiation signals (16, 28). Thus, it is intriguing that sustained Nur77 overexpression in the mixed BM chimeras abrogated NKT2 cell development, whereas NKT17 subset development was intact. It is conceivable that strong TCR signals induces other factors and signaling modules apart from Nur77 or, alternatively, that Nur77 plays distinct roles in different iNKT cell subsets, or both. This function of Nur77 in effector differentiation of iNKT cells is in striking contrast to its role in the development and differentiation of conventional T cells.

Nur77 controls the induction of self-tolerance at multiple levels, including negative selection of developing iNKT cells, as discussed above. Furthermore, Nur77 induces an exhausted phenotype in iNKT cells as the developing cells express PD1, which is associated with iNKT cell anergy induction (61–63). *Nr4a* family transcription factors (TFs) induce T cell hyporesponsiveness. The Nr4a TF-binding motif, ~23 kb upstream of the *Pdcd1* transcription start site, is highly enriched in several models of T cell exhaustion but is absent in *Nr4a* TKO mice. Similarly, Nur77 expression is strongly correlated with HeK4me3 (accessible chromatin) marks near tolerance-related genes (24–26). These findings in conventional T cells suggest that induction of Nur77 within thymic iNKT cells may program a tolerant or exhausted phenotype. Enhanced expression of FR4, NRP1, and CD244—markers of T/iNKT cell tolerance—on iNKT cells overexpressing Nur77 further supports this notion. iNKT cell tolerance so induced is distinct in that this process occurs in the thymus rather than in the periphery as seen in conventional T cells.

iNKT cells in mouse strains and derived chimeras that overexpressed Nur77 were hyporesponsive to agonistic stimulation in vivo with αGC. CD4$^+$ T cell precursors from *Nr4a* TKO mice produce substantially high levels of IL-4 compared with wild-type mice (19, 71). Furthermore, *Nr4a* TKO CD8$^+$ chimeric antigen receptor (CAR) T cells have enhanced IFN-γ production compared with their wild-type counterparts. A Nur77-binding motif in the *Ifng* locus is enriched in *Nr4a*-TKO CD8$^+$ CAR T cells (24, 26), suggesting that Nur77 directly binds to regulatory

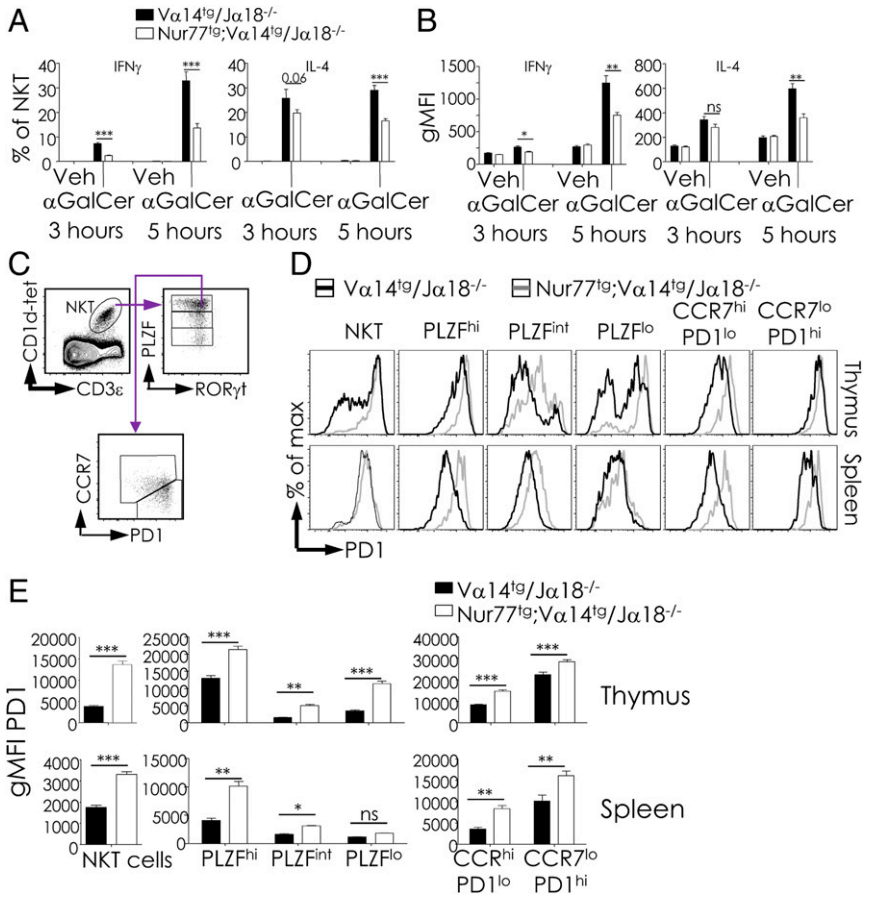

Fig. 6. Exhausted and functionally impaired iNKT cells emerge in mixed BM chimeras. (*A* and *B*) Vα14^{tg}/Jα18$^{-/-}$ and Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ mixed BM chimeras were injected i.p. with vehicle or αGC, and spleens were harvested at the indicated times after injection. Splenocytes were surface-stained to identify iNKT cells and then stained with mAbs to detect intracellular IFN-γ and IL-4. (*A*) Bar graphs depict mean ± SEM of the proportion of IFN-γ$^+$, IL-4$^+$, and IFN-γ$^+$IL4$^+$ iNKT cells. *n* for 3 h: Vα14^{tg}/Jα18$^{-/-}$, vehicle = 3, αGC = 3; Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$, vehicle = 5, αGC = 7; *n* for 5 h: Vα14^{tg}/Jα18$^{-/-}$, vehicle = 5, αGC = 6; Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$, vehicle = 7, αGC = 9. (*B*) Bar graphs depict mean ± SEM gMFI of IFN-γ (*Left*) and IL-4 (*Right*). Data are representative of three to five independent experiments. *n* as in *A*. (*C*–*E*) Thymocytes and splenocytes from Vα14^{tg}/Jα18$^{-/-}$ ($n = 5$) and Nur77^{tg};Vα14^{tg}/Jα18$^{-/-}$ ($n = 5$) mixed BM chimeras were surface-stained to identify iNKT cells and expression levels of CCR7 and PD-1. They were then stained with specific mAbs for PLZF and RORγt. (*C*) Contour plots showing gating strategy to identify PLZF^{HI}, PLZF^{INT}, and PLZF^{LO} (all RORγt^{NEG}) iNKT cells in the thymus and spleen. The PLZF^{HI} population was further gated to identify CCR7^{HI}PD1^{LO} and CCR7^{LO}PD1^{HI} NKT2 cells. PD-1 expression was monitored on the indicated iNKT cell populations. (*D*) Overlay histograms are representative of two independent experiments. (*E*) gMFIs of PD-1 expression. Data are mean ± SEM from two independent experiments. *n* as above. ns, not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$.

regions and represses expression. In addition, other cytokine gene loci, such as *Il21* and *Tnfa*, showed enrichment for Nur77 without a direct Nur77-binding motif, suggesting that Nur77 can induce the expression of negative regulators of cytokine genes and thereby control T cell responsiveness (24, 26, 71).

Finally, Nur77 overexpression induced Foxp3 expression on a subset of iNKT cells and the emergence of cells resembling conventional Tregs. We predict that Nur77-induced cell death in the thymus induces transforming growth factor-β, which is known to induce Foxp3 expression on iNKT cells (72). Furthermore, IL-10–producing Foxp3$^+$ regulatory iNKT cells are produced in a paracrine indoleamine 2, 3-dioxygenase–dependent manner in an acute liver injury model (73). Thus, multiple mechanisms ensure the induction of self-tolerance in this self-reactive iNKT cell lineage.

In summary, this study has unveiled a critical, yet hitherto unknown, role for Nur77 in iNKT cell development, effector subset differentiation, and function. Nur77 controls negative selection by inducing caspase-3–mediated cell death and iNKT cell hyporesponsiveness as a means of self-tolerance induction. It does so via cell-intrinsic and -extrinsic mechanisms that are distinct from its role in conventional T cell development. These findings suggest a control mechanism to ensure that iNKT cells do not go rogue to cause autoimmune or autoinflammatory diseases, and identify Nur77 as a target for clinical intervention should that occur.

Materials and Methods

Mice. Age-matched 8- to 10-wk-old mice were used for all the experiments described herein. B6-Nur77^{tg} (Nur77^{tg}) (17) and B6-Jα18$^{-/-}$ (Jα18$^{-/-}$) (74) mice were generous gifts from A. Winoto and M. Taniguchi, respectively. B6-Vα14^{tg} (Vα14^{tg}) (36) and B6-CD1d1$^{-/-}$ (CD1d$^{-/-}$) (75) mice have been described previously. B6 (C57BL/6J) mice were purchased from The Jackson Laboratory. Nur77^{tg} mice were crossed with B6 or Vα14^{tg} mice to obtain Nur77^{tg} or Nur77^{tg};Vα14^{tg} mice, respectively. A single copy of each transgene was maintained for the studies. All mouse strains generated by us for this study are available from the corresponding author on request.

All procedures performed on mice in this study were approved by the Institutional Animal Care and Use Committee at Vanderbilt University School of Medicine.

Reagents and Experimental Procedures. The step-by-step protocols followed in this study have been reported previously (45). More details are provided in *SI Appendix*.

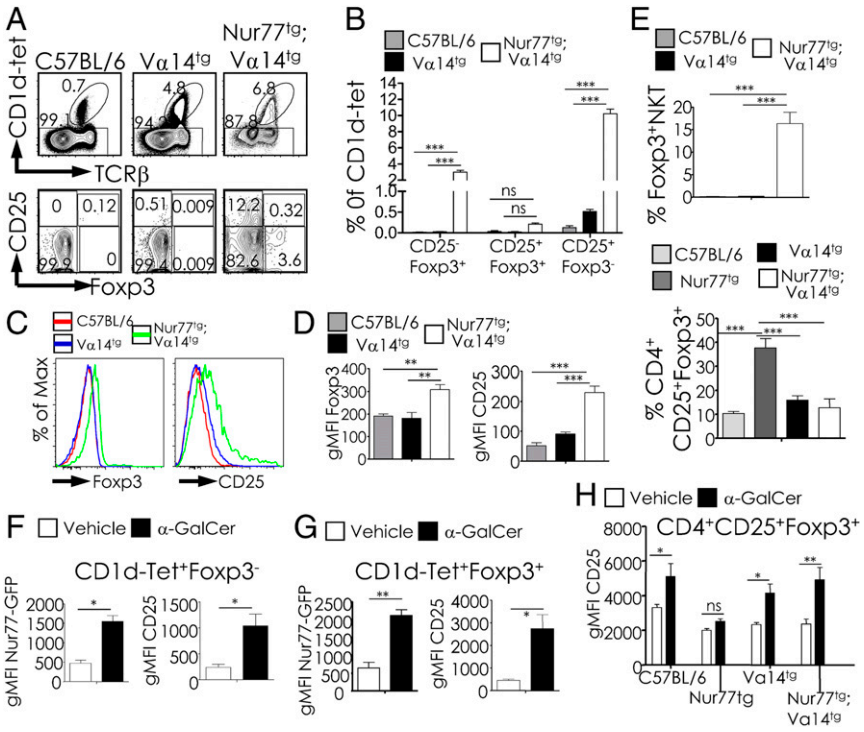

Fig. 7. Nur77 overexpression induces a Foxp3$^+$ iNKT cell subset that responds to αGC stimulation. (*A* and *B*) Thymic iNKT cells from C57BL/6 ($n = 5$), Vα14^{tg} ($n = 5$), and Nur77^{tg};Vα14^{tg} ($n = 7$) mice were identified as before, and surface CD25 and nuclear Foxp3 expression levels were monitored. (*A*) Contour plots are representative of three independent experiments. (*B*) Bar graphs show cumulative mean ± SEM of the percentage of CD25^{NEG}Foxp3$^+$, CD25$^+$Foxp3$^+$, and CD25$^+$Foxp3^{NEG} within iNKT cells. *n* as in *A*. (*C* and *D*) Constitutive Foxp3 and CD25 expression by iNKT cells from mice as in *A*. (*C*) Overlay histograms are representative of three or four independent experiments. (*D*) gMFIs of Foxp3 (*Left*) and CD25 (*Right*) expression on thymic iNKT cells from mice as above. Data are cumulative mean ± SEM from three independent experiments. *n* as in *A*. (*E*) Splenocytes from C57BL/6 ($n = 5$), Nur77^{tg} ($n = 3$), Vα14^{tg} ($n = 4$), and Nur77^{tg};Vα14^{tg} ($n = 4$) mice were stained to identify iNKT cells and CD4$^+$ T cells as before. CD25 and Foxp3 expression on iNKT cells and CD4$^+$ T cells were monitored. Bar graphs show cumulative mean ± SEM of the percentage of Foxp3$^+$ iNKT cells (*Upper*) and CD4$^+$CD25$^+$Foxp3$^+$ T cells (*Lower*). (*F* and *G*) Inducible Nur77^{GFP} (*Left*) and CD25 (*Right*) expression on Foxp3^{NEG} (*F*) and Foxp3$^+$ (*G*) splenic iNKT cells from Nur77^{tg};Vα14^{tg};Nur77^{GFP+} mice injected i.p with vehicle ($n = 2$) or αGC ($n = 3$). Bar graphs are cumulative mean ± SEM. (*H*) Splenocytes from C57BL/6, Nur77^{tg}, Vα14^{tg}, and Nur77^{tg};Vα14^{tg} mice injected with vehicle or αGC were stained to identify CD4$^+$ T cells as before. CD25 expression on CD4$^+$ T cells was monitored. gMFI of CD25 expression by CD4$^+$CD25$^+$Foxp3$^+$ cells is shown. Data in the bar graphs are cumulative mean ± SEM. C57BL/6: vehicle, $n = 5$; αGC, $n = 5$; Nur77^{tg}: vehicle, $n = 3$; αGC, $n = 4$; Vα14^{tg}: vehicle, $n = 4$; αGC, $n = 4$; Nur77^{tg};Vα14^{tg}: vehicle, $n = 4$; αGC, $n = 5$. ns, not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$.

Data Availability. All data are presented in the main figures and supplemental figures. Raw data and replicates are available from the corresponding author on request.

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